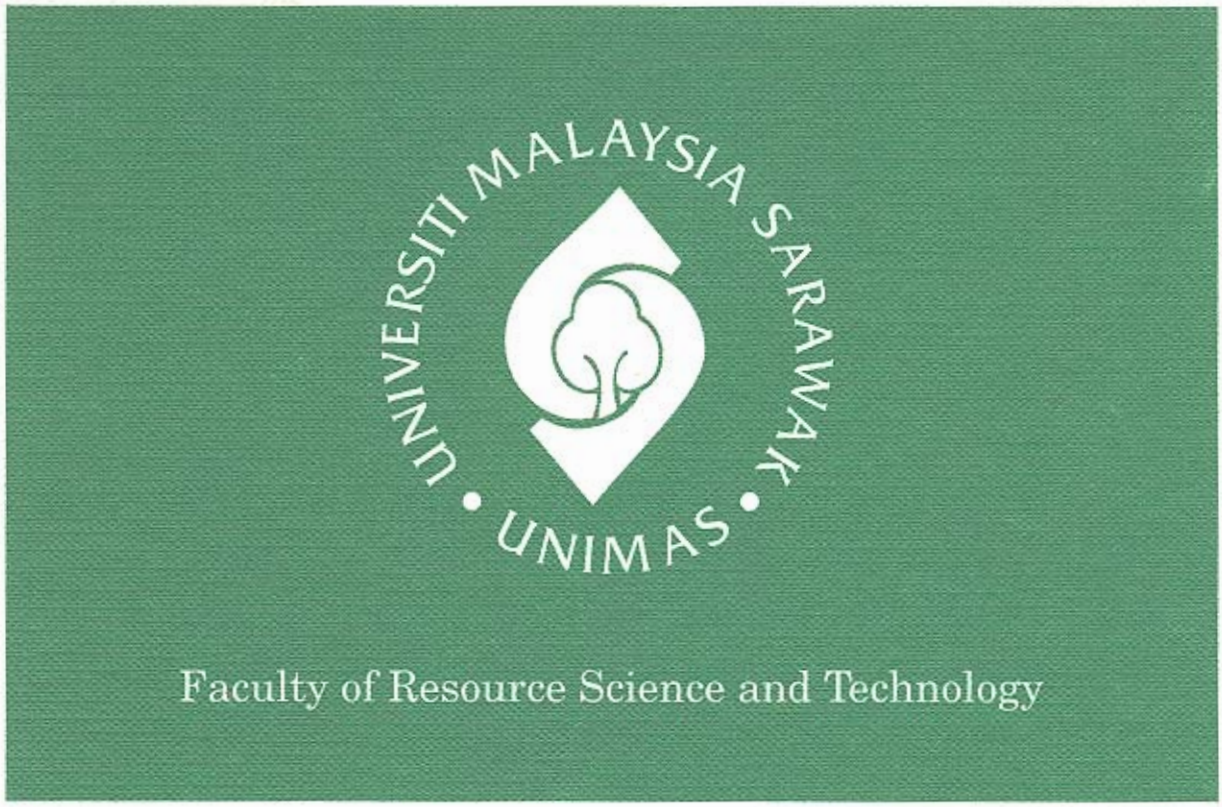

POLYMERASE CHAIN REACTION - RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (PCR-RFLP) ANALYSIS OF 16S rRNA GENE IN *Myotis ridleyi*

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ABSTRACT

Variation in the restriction sites and in the length and site of polymorphisms of 16S rRNA gene from eight *Myotis ridleyi* were assessed using polymerase chain reaction-restriction of fragment length polymorphisms (PCR-RFLP) analysis. They were subjected to four different restriction enzymes. These samples were collected from four different geographical areas: Taman Kinabalu, Poring, Danum Valley and Gunung Serapi. A RE map and dendrogram were constructed based on the fragment size and patterns that were produced by the limited set of enzymes. The different individuals showed variation and polymorphism in their 16S rRNA gene when analyzed by PCR-RFLP. Dendrogram reviewed that the individuals from same habitat tend to cluster closer to each other. This happens when mutation takes place and cause the deletion of the RE recognition site. The finding is best if confirm with sequencing result. However, more samples should be studied and more enzymes should be utilized to give better comparison patterns. This is needed for a more convincing and statistically significant conclusion.

Key Words: Polymorphism, RFLP, Mitochondrial 16S rRNA gene, restriction endonucleases (RE).

ABSTRAK

Analisasi 'PCR- RFLP' telah dijalankan ke atas gen 16S rRNA mitokondrial Myotis ridleyi untuk mengkaji sama ada individu ini menunjukan perbezaan saiz dalam penghasilan pecahan DNA. Lapan sampel ini akan dipotong dengan empat enzim pembatas. Bahan kajian ini dikumpul dari empat kawasan geografi yang berbeza iaitu dari Taman Kinabalu, Poring, Danum Valley dan Gunung Serapi. *Saiz dan corak DNA yang dihasil telah digunakan untuk membina 'restriction map' dan juga 'dendrogram'. Jalur DNA individu yang terhasil dari lokasi berbeza berkemungkinan menunjukan pembezaan dalam saiz. Ini mungkin disebabkan mutasi yang berlaku dan menyebabkan 'restriciton site'dihapuskan. 'Dendrogram' menunjukan individu dari lokasi berhampiran dan habitat sama cenderung untuk berkelompak dalam satu kumpulan . Selain itu, penemuan ini adalah perlu disokong dengan keputusan 'sequencing'. PCR-RFLP adalah kaedah yang baik untuk menjalankan eksperimen ini. Walaubagaimanapun, lebih banyak enzim perlu digunakan demi membekalkan 'fragment pattern' yang lebih baik untuk perbandingan.Lebih banyak sampel diperlukan untuk menghasilkan data dalam membuat keputusan yang lebih sah dari segi statistik.*

Kata kunci: 'Polimorfisme', 'PCR-RFLP', gen 16S rRNA mitokondrial, enzim pembatas

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CHAPTER 1

INTRODUCTION and LITERATURE REVIEW

1.1.1 Microchiroptera

Myotis ridleyi is placed in the Chiroptera order and also in the suborder of microchiroptera (insectivorous bats). The order of the Chiroptera is the largest group of mammals in Malaysia. It is recorded that 40% of the 28 species of mammals are Chiroptera (Yong, 1998). This order could be further divided into 2 suborders: Megachiroptera (fruit bats) and Microchiroptera (insect bats) (Payne *et al*, 1985; Findley, 1993). Fruit bats are considered to have relatively good eyesight, but they still rely primarily on sense of smell when they are in search of food. Meanwhile insect bats use high frequency echolation to locate obstacle and preys while flying in total darkness (Salleh, *et al*, 1999; Vaughan, 1986 and Yong, 1998).

Francis (2001) also stated that *Myotis* is the most widespread genus of microbats. The *Myotis* genus has three distinguishing morphological characters. Normally, *Myotis* have three upper and lower premolars, tall distinctly shaped ears that are narrow near the top, and the long tragus inside that tapers to the tip. However, the morphology of some of the *Myotis spp* appear to be very similar, some are virtually identical in external appearance (Nagorsen, 1996). Hence, researchers need to utilize molecular techniques, (nucleotide sequence), to increase the accuracy of discriminating these bats species.

1.1.2 *Myotis ridleyi*

Myotis ridleyi is a dark coloured bat. These dark coloured bats are generally characterized by their small body size. The upper body part is dark grey-brown whereas the lower part is paler and greyer. Though they are small in size they have relatively heavy body weight, about 4 to 6 g. Besides this, they have typical tragus-shape *Myotis* ears (Payne *et al*, 1985). Based on the 14 collected specimens captured at Sepilok and Witti range in Sabah, they differ from other *Myotis spp* by having only 2 upper and lower premolars, as recorded by Payne *et al*, (1985). Figure 1 shows the distribution of *Myotis ridleyi* in IndoMalayan region (Corbet and Hill, 1992).

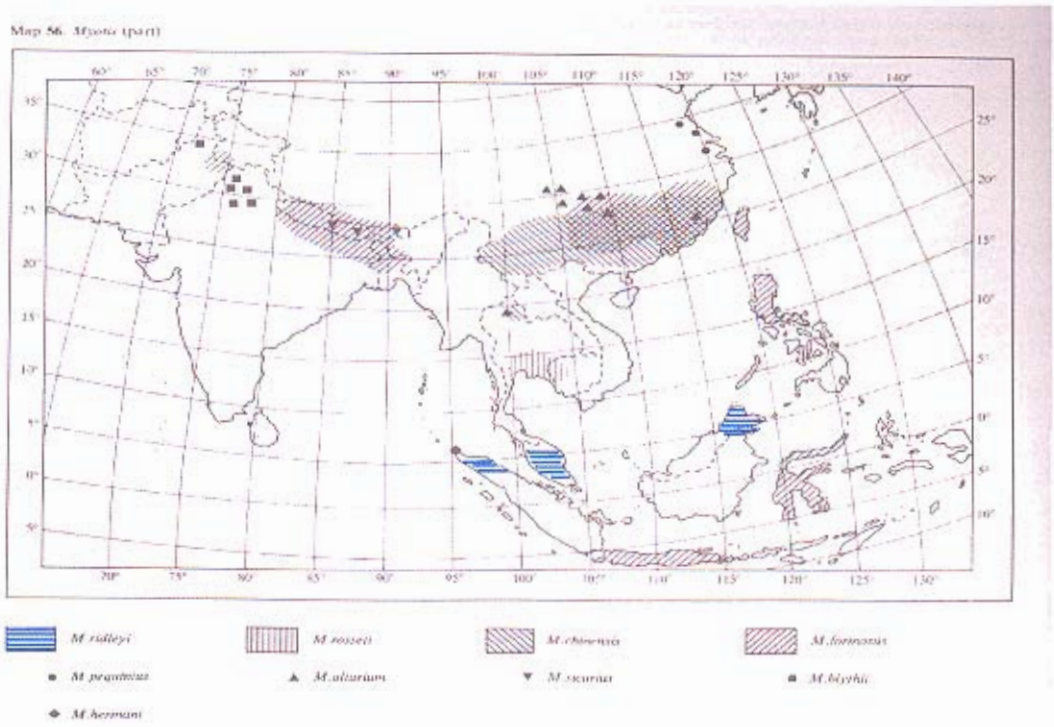

FIGURE 1: Distribution of *Myotis ridleyi* in IndoMalayan region
Adapted from "The mammals of the IndoMalayan Region (Corbet and Hill, 1992).

1.1.3 Mitochondrial genome

Mitochondrial genome of multicellular animals range from 15000 to 19500 bps in length (Hoelzer and Dover, 1991). Generally, it encodes 2 rRNAs, 22 tRNA and 13 polypetides. The rRNA could be further divided to 12s rRNA, (500bps) and 16s rRNA, (700bps) whereas 22 tRNA is required for mitochondrial protein synthesis (Li and Graur, 1991). The lowest mitochondrial DNA (mtDNA) substitution rates occur in tRNA and rRNA genes while mtDNA protein genes evolve at about twice the rate of the tRNA and rRNA genes (Hoelzer and Dover, 1991).

Besides this, the mutation rate of mtDNA is higher than nuclear DNA (Li and Graur, 1991; Gilham, 1994). This is probably due to the insufficient DNA repair system in the mtDNA (Li and Graur, 1991). Therefore, these unique features of mtDNA can be used to test the population level studies such as short-term evolution and migrations among the mammals (Hoelzer and Dover, 1991; Li and Graur, 1991; Silva and Russo, 2000). Based on previous studies, (Doyle, 1992; Hillis and Davis, 1992), that involve both molecular and morphological data, better descriptions and interpretations of biological diversity occurs if compared to those which only utilize one approach

Currently, mitochondrial DNA (mtDNA) is the most widely studied molecule for population genetics researches (Silva and Russo, 2000). It is because mitochondrial DNA is much smaller if compared to nuclear DNA. In addition, it is inherited maternally, that is through the transmission of the egg cytoplasm (Hoelzer and Dover, 1991). Therefore, it is easier to be investigated experimentally. Furthermore, animal mitochondrial genome is the most characterized among any group of organism (Avise *et al,* 1990). Hence, it is easier to retrieve genetics information or nucleotides sequences from the resources for references and comparison purposes.

In addition, the numbers of mitochondria (thus, mtDNA) increase with the energy requirement of the tissues (eg. wing muscle tissues of the bat). Therefore, the amount of tissues required for the laboratory tests can be reduced (Onarici and Sumer, 2002). This is because small amount of the muscle tissues will already provide sufficient copy of mtDNA for the research. It is important especially when the DNA resources are limited. Most of the researches done using mitochondrial genes were to study the interrelationships of populations, families and also the orders (Moritz *et al*, 1987; Lamb *et al*, 1994).

Numerous studies on Microchiroptera have been conducted especially in America, Europe and Africa. Most of these studies were used to address the molecular systematics and phylogenetic relationships of the Microchiroptera. Among the researches done, two studies had successfully resolved the phylogenetics relationship of Mormoopid bats and also Pleotino bats based on mitochondrial ribosomal sequences (Van Den Bussche *et al*, 2002; Hoofer and Van Den Bussche, 2001). Besides this, other researches on the molecular systematic of *Myotis occultus* and *Myotis lucifungus* have been carried out by sequencing of the mitochondrial cytochrome b and cytochrome oxidase II genes (Piaggio, 2002).

In Borneo, the studies on Microchiroptera identification and their distribution so far has only been based on morphological characterization (Payne *et al*, 1985). However recently, some comprehensive studies utilizing molecular approaches have been carried out.

1.1.4 Mitochondrial 16S rRNA gene

16S rRNA gene is one of the highly conserved genes in the mammals' mitochondrial genome (Onarici and Sumer, 2002). This gene is commonly used as a marker for genotyping and phylogenetics analysis (Hoofer and Van Den Bussche, 2001). For example, such studies that is based on 16S rRNA gene were not only carried out on animal but has also been carried out earlier in cockroaches, black flies, mosquitoes and other insects as well (Houche and Atole, 2000). One of the reasons is because copies of 16S rRNA genes are abundant in the cell. So, the number of cells required for experimental studies is reduced (Onarici and Sumer, 2002). Hence, 16S rRNA gene marker was utilized for PCR-RFLP analysis to characterize DNA polymorphism in *Myotis ridleyi* in this study.

1.1.5 Restriction Endonucleases

Restriction Endonucleases (RE) are enzymes that cleave DNA molecule at specific sequences depending on types of enzymes used. The recognition sites of the enzyme are about 4 to 6 base pairs in length. Some RE make staggered cuts but some make double stranded scission that produced blunt-ended. For the staggered cuts, single-stranded overhangs will be produced or sticky ends. These staggered ends can rejoin through base pairing with complementary DNA fragment cut by the same RE (Walker and Gaastra, 1983 and Weaver, 1999).

1.1.6 Polymerase Chain Reaction -Restriction of Fragment Length Polymorphisms (PCR-RFLP)

PCR-RFLP is the technique used to differentiate between the same or different species of organisms by analyzing the patterns derived from cleavage of their DNA via RE. Different length of fragments can be obtained when the genomic DNA from different species are subjected to the same RE. Hence, the presence or absence of the fragment at recognition sites will be useful for identifying species and to differentiate them from one another (Dowling *et al*, 1996). The different length of fragments produced can be due to mutation that occurs within the RE recognition sequence of the same species (different individual). Base substitutions, insertion or deletions event are the most likely examples of the mutation in this study (Avise, 1994; Dowling *et al*, 1996). Thus, it gives rise to the polymorphism of the DNA fragments that are subjected to RE. For example, variation and polymorphism of mitochondrial DNA has been reported among four laboratory colonies of weevils that were collected from different geographical and host plant origin by using RFLP analysis (Roehrdanz and North, 1992).

PCR-RFLP analysis was used in this study because it is one of the rapid and also cheaper means of large scale analytical approaches when compared to DNA sequencing strategy. In addition, it is not a technically demanding technique. This technique only requires suitable target DNA fragment (Silva and Russo, 2000). Moreover, the only effort required is the testing of a number of RE enzymes in order to establish which one will show reproducible polymorphic pattern (Dowling, *et al*, 1996). Therefore, the result obtained in this study will be a great benefit for the preliminary analysis of polymorphism and identification of genetic marker analysis.

1.2 Hypothesis

In this study, two hypotheses concerning the RE site polymorphic on DNA fragment among the eight *Myotis ridleyi* were proposed. The null hypothesis would be that the restriction site of the 16s rRNA genes of the *Myotis ridleyi* from different geographical locations are the same. Meanwhile the alternative hypothesis would be not all the restriction site of the 16S rRNA genes of *Myotis ridleyi* from the different geographical area are the same.

1.3 Objectives

The main objective of this project is to study the polymorphism fragment length patern of mitochonrial 16S rRNA gene among the Myotis spp between four different localities when subject to restriction enzymes. Besides this, this study is also conducted to determine the appropriate Restriction Endonucleases (genetics marker) for 16S rRNA genes on *Myotis ridleyi*. Then, the information derived from the RE digestion, (fragment sizes and pattern), is used to construct a RE map and also a crude dendrogram.

CHAPTER 2

MATERIALS and METHODS

2.1 Preserved Samples taken from previous years

Eight samples of *Myotis ridleyi* were taken from the FSTS animal museum for this study. These samples were collected from four different geographical areas in Sabah (four from Poring, one from Taman Kinabalu and one from Danum Valley) and Sarawak (one from Gunung Serapi), (Table 1 and Figure 2).

TABLE 1: Samples for this study

Samples		Tissue/ Skin/ Genomics DNA	Year Collected	Localities	Collectors (as labeled on bottles)
Myotis ridleyi A	TK 001	Tissue & Skin	1996	Mt Kinabalu,Sbh	Dr. M. Taj., Isa. & Bc.
Myotis ridleyi B	MTA 96328	Tissue & Skin	1996	Mt. Serapi, Srwk	Dr. M. Taj.
Myotis ridleyi C	PR 055	Genomic DNA	2000	Poring, Sbh	FRST students
Myotis ridleyi D	PR 056	Genomic DNA	2000	Poring, Sbh	FRST students
Myotis ridleyi E	PR 057	Genomic DNA	2000	Poring, Sbh	FRST students
Myotis ridleyi F	PR 058	Genomic DNA	2000	Poring, Sbh	FRST students
Myotis ridleyi G	PR 059	Genomic DNA	2000	Poring, Sbh	FRST students
Myotis ridleyi H	F# 21	Tissue & Skin	1996	Danum V. Sbh	Dr. M. Taj., Isa. & Bc.

Sbh, Sabah; Mt, Mountain; Srwk, Sarawak; Danum V, Danum Valley.
Dr. M. Taj: Dr Mohd Tajuddin, Isa: Isa Sait, Bc: Besar Ketol.
The number for each individual represents collection numbers from the previous sampling done in Sabah and Sarawak and were used as cross references between lab result and previous studies.

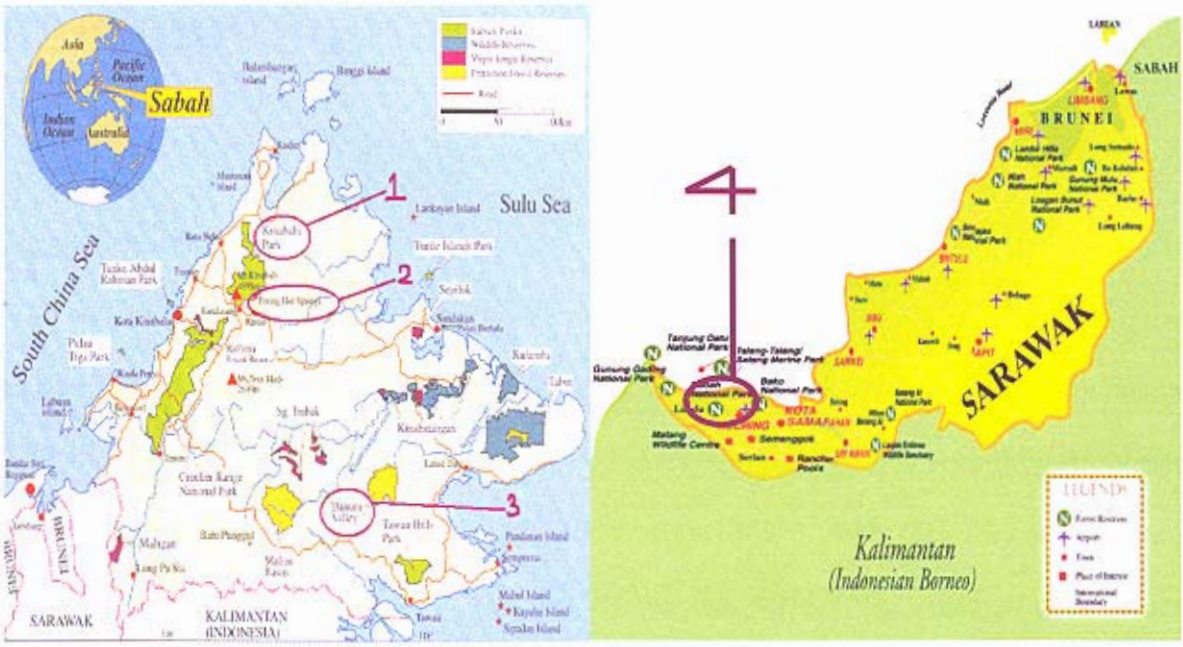

FIGURE 2: The geographic origin of the individual of the samples collected.
1. Taman Kinabalu, Sabah 2. Poring, Sabah 3. Danum Valley, Sabah 4. Gunung Serapi, Kubah, Sarawak
Adapted from the World Wide Web:
1.http://impressions.com.my/sabah/sabahinfo/sabahmap.htm
2.http://www.sarawakmice.com/map/

2.2 METHOD

2.2.1 Cutting of tissue samples

Tools and kits for cutting samples; scalpel blades, Petri dishes, lighter, Bunsen burner, forceps, 70% ethanol, ddH_2O, 2 Azlon containers (5mL & 500 mL), and cryogenic vials,(1.5ml or 5ml) were prepared before hand. They were in sterile condition. The cryogenic vials were filled with about 3mL of the 70% ethanol for samples storage.

Before the samples were cut, 70% of ethanol was used to clean the working surface. Then, the samples of interest; TH007, MTA 96328, and F# 21, were taken out from the cupboard of the mammal section. Both the skin and the tissue were cut for DNA

extraction purposes. After cutting the tissue, they were kept in the cryogenic vials at -20°C until required for use.

2.2.2 DNA Extraction:

Total genomic DNA extraction was carried out following the protocol of the Viogene Blood and Tissue Genomic DNA Miniprep System (Viogene U.S.A., GG 1001). Firstly, about 30mg of the tissue from each sample were cut and the tissues were washed with the PBS buffer solution. They were then finely minced using sterile scalpel blade. After this, they were placed into the 1.5mL sterile Eppendorf tube. The hair on the skin surface was removed first before mincing. Then, 200µL of LYS Buffer was added to the tube. The tissues were later homogenized by using syringe.

Secondly, 20µL of the Proteinase K was added to each of the minced tissues. The reactions were vortex for 20 seconds. After this, they were incubated for 1 hour at 60°C in order to lyse the tissues completely. The tissues were transferred to a heater block for 20 minutes for incubation at 70°C. At the same time, 100µL of ddH_2O was preheated for the DNA elution step.

Thirdly, 200µL of EX buffer was added in to each of the mixtures and again mixed by vortexing for 20 seconds. After the mixtures were well mixed, they were incubated for another 10 minutes. Then, another 210µL of absolute ethanol was added into each mixture, followed by vortexing.

Fourthly, the B/T Genomic DNA Mini Columns were placed into the 2ml collection tubes. All the mixtures from the 1.5µL were then transferred to the new 2µL collection tubes. Then the tubes with the mixture were centrifuged at 8000rpm for 2 minutes. After this, the columns were placed into another new 2µL collection tubes. The columns were washed twice with 500µL WS

buffer via centrifugation at 8000rpm for 2 minutes. The flow-through was discarded each time after the centrifugation. For the second wash, the columns were spun at full speed for another 2 minutes to remove the ethanol residue.

Fifthly, the columns were placed into new 1.5µL tubes. Then, the DNA were eluted by using 80µL of preheat water. The columns were left to stand for about 2 to 3 minutes to allow complete elution of DNA. The eluted DNA was then stored at 4° C or -20° C until further use.

For the samples PR055, PR056, PR057, PR058 and PR059, genomics DNA were ready for further use or analysis.

2.2.3 Polymerase Chain Reaction (PCR):

The partial 16S rRNA genes from each sample were amplified using PCR strategies. The expected size of 16S rRNA genes that were generated with the primers is approximately 600 basepairs. These 16S rRNA primers were available in the laboratory. The primers sequences are as below:

16S U 5' – ACCCGGGGATCCCTGTTTACCAAAAACATCACC – 3'

16S L 5' – CCGGATCCCCGGCCGGTCTGAACTCAGATCACG – 3'

These primers were synthesized by OPERON technology, Inc (USA). The PCR cocktail reaction was prepared in laminar flow hood. A master mix, which contains all the reagents except *Taq* polymerase and DNA template, was prepared. The master mix reaction was vortexed and centrifuged for 20 seconds. Then, the master mix solution was aliquot into different tube according to the right proportion of volume. The DNA template and *Taq* polymerase were added separately into each tube. During the reaction mixture preparation process, *Taq* polymerase was

kept on ice. It was only added in at the last step of master mix preparation. Table 2 shows the PCR set-up employed.

TABLE 2 : PCR reaction cocktail for one reaction

Solutions / reagents	Volume (µl)
10X PCR buffer	5.0
25 Mm $MgCl_2$	3.0
10 Mm dNTPs mix	1.0
25 pmole/ µl 16 SU primer	2.0
25 pmole/ µl 16 SL primer	2.0
Taq Polymerase (1 / µl)	2.0
Template DNA	3.0
UP H_2O	32.0

Besides the components listed in Table 2, negative control was prepared to monitor authenticity of results. Component of the mixture reaction for negative control is the same as shown in Table 2, except that it does not contain the DNA template and sterile Ultra Pure (UP) H_2O was used instead. The PCR parameters of amplification are showed in Table 3:

TABLE 3 : PCR Amplification parameters

Process	Temperature (° C)	Time
Initial denaturation	96 ° C	2 minutes
Denaturation	94 ° C	40 seconds
Annealing	56 ° C	45 seconds
Extension	72 ° C	45 seconds
Final Extension	72 ° C	10 minutes

The numbers of amplification cycles are 35.

2.2.4 Gel Electrophoresis:

The horizontal agarose gel system was used to estimate the yield and purity of the PCR products. One percent of 40µL agarose gel was prepared. Three micro litre (µl) of PCR products were mixed with 1µL loading dye and 2µL UP H_2O and then, load them into each well. Electrophoresis was carried out at 102 Volts for about 30 minutes. Then the gel was immersed into the Etedium Bromide for 30 minutes to enable fluorescent visualization.

2.2.5 PCR Products Purification:

PCR products purification process was carried out in accordance with the protocols of the manufacturer (Gel-M™ Gel Extraction System and also the PCR-M™ Clean UP system).

2.2.5.1 Gel Extraction System:

Before the gel extraction step, 2% of the 50µL agarose gel of four wells (maximum) was prepared. Next, 100µL of the PCR product with 10µL of gel loading dye from each sample were loaded into each well. Electrophoresis was at 105 V for 45 minutes. After this, the gel was immersed in the Ethidium Bromide for 30 minutes. Then, visualize the gel on the UV box. The gel slices with the desired DNA fragments were excised using the sterile scalpel blade. The scalpel blade was sterilized with 95% ethanol each time before cutting the DNA fragment from different samples.

Gel slices with the desired DNA bands were then put into four different, sterile 1.5µL Eppendorf tubes. Subsequently, 500µL of the GEX buffer was added to four different tubes. This was followed by incubation at 60° C for 10 minutes until the gel slices were completely dissolved.

The tubes were inverted every two minutes to enhance the dissolving process. After the gel slices were completely dissolved, these tubes were left at room temperature to cool.

Four Gel-M™ Columns were placed into the four Collection tubes and 600µL of the dissolved gel mixture from each tube were loaded into the four different columns. Then, the dissolved gel mixtures were spun at 8000rpm for 1 minute. After this, the column was washed with 500µL WF Buffer and then the column was subjected to centrifugation for 1 minute. The flow-through obtained was discarded after centrifugation.

Seven hundred µL of WS buffer was added and the mixture was centrifugated at 8000 rpm for another one minute. All the flow-through was discarded again. Next, the columns were centrifuged at maximum speed, 14000rpm for 4 minutes in order to remove all the residual ethanol. After this, the columns were placed into another new, sterile 1.5µL Eppendorf tubes. Thirty-three µL of the ddH_2O was added onto the center of the membrane later. For high recovery of the product, the ddH_2O was added to the center of the membrane and steps were taken to made sure that it is completely dissolved.

Finally, the columns were left to stand for 2 minutes at room temperature and this was followed by centrifugation. The eluted DNA was stored at -20°.

2.2.5.2 PCR Clean Up

The five PCR products (PR 055, PR 056, PR 057, PR 058 and PR 059), were cleaned using the PCR Clean Up, Spin Method. The protocol described below was based on manufacturer's recommendation.

First, 50 µl of each of the PCR product was pipetted into a new 1.5 µl Eppendof tube. Then, 500 µl of the PX buffer was added and the reaction was mixed by vortexing. Secondly, PCR-M Columns were placed into a 2 µl Collection tubes. All the mixtures from first step were transferred to the new tubes. Then, centrifugation was carried out at 8000rpm for 1 minute. The flow through was discarded after this.

Thirdly, the columns were washed with 500 µl WF Buffer by centrifugation at 8000rpm for 1 minute. After this, the flow through was discarded as well. Then, the columns were spun again at full speed for another 4 minutes to remove all the residual ethanol.

Fourthly, the columns were placed into new 1.5 µl Eppendorf tubes. Thirty µl of the preheat UP H_2O was added into each tube to elute DNA. Then, columns were left to stand for about 2 minutes. After this, the tubes together with the columns were spun at 8000 rpm for 2 minutes. Eluted DNA was stored at -20°C.

2.2.6 PCR- Restriction of Fragments Length Polymorphism (RFLP):

During RFLP, four specifically picked Restriction Endonuclease enzymes; *Alu* I, *Bsu* RI, *Mbo* I, and *Taq* I were used to cut the amplified 16S rRNA gene (Table 4). The 10µl RFLP cocktail mixture was prepared as shown in Table 5. These enzyme digestion mixtures were incubated for six to nine hours at optimum temperature.

TABLE 4: Restriction Endonuclease and the Reaction Condition:

Enzyme	Restriction Sites	Buffer	Unit for overnight Incubation (u/μg per unit)	Optimum Temperature (°C)	Inactivation Temperature (°C)
Alu I	5'-A G^C T-3' 3'-T C^G A-5'	Buffer B (Promega)	0.1	37°C	65°C, 20 mins
Bsu RI	5'-G G^C C-3' 3'-C C^G G-5'	Buffer R (Fermentas)	0.1	37°C	80°C, 20mins
Mbo I	5'-^G A T C -3' 3'- C T A G^-5'	Buffer R (Fermentas)	0.1	37°C	65°C, 20 mins
Taq I	5'-T^C G A-3' 3'-A G C^T-5'	Buffer E (Fermentas)	0.1	65°C*	No

*under paraffin oil in a capped vial.
(Information is adapted from the *Alu*I, *Bsu* RI, *Mbo*I, *Taq*I, Fermentas Life Science Catalog)

Among the four RE enzymes, *Alu* I, *Bsu* RI, *Mbo* I and *Taq* I, that were used in the experiments, both the *Alu* I and *Bsu* RI enzyme are not sensitive toward methylation system whereas both *Mbo* I and *Taq* I are sensitive to Dam methylation. DNA methylations are mostly found in the bacteria system. They play very important role in the restriction-modification (R/M) system, DNA replication as well as the post replicative mismatch repair in the bacteria (Dryden, 1999). Bacteria that have the R/M system will express a methylase and cognate restriction endonuclease that can recognize the same target site. The presence of the methylation system normally will protect the bacteria chromosome against foreign RE enzyme (Roberts and Macel, 2001). Hence, the activities of the RE enzyme that are sensitive toward Dam and Dcm metyhlation of one particular nucleotide in the recognition sequence could be inhibited.

Samples were subjected to single and also double digestions. During single digestion, only one enzyme was mixed to the reaction mixture to cleave the samples whereas double digestion involved two restriction enzymes. The different DNA fragments that were produced through the restriction enzymes activities were separated on agarose gel. The results obtained

were used to construct a linear restriction enzymes map. Restriction map is a description of restriction endonuclease cleavage sites within a piece of DNA (Strachan, 1992).

Based on the scoring from the PCR-RFLP fragments patterns, a dendogram was constructed using the NT-sys software too. Dendrogram is a branches diagram or tree that shows evolutionary distance along its horizontal axis. UPGMA is the simplest and most straightforward method of a tree construction. This method uses a sequential clustering algorithm. It identifies the local topological relationship according to their genetic similarity. The individuals that were grouped in the same cluster have higher similarity in their genetics similarity.

TABLE 5: 10µL RFLP reaction cocktail (one reaction)

Solutions / Reagents	Volume (µl)
ddH_2O	6.65
RE Buffer	1.0
BSA	0.1
Restriction Enzyme	0.25
DNA	2.0

Incubation time: 6-9 hours

The master mix solution that contains all the reagents (EXCEPT DNA template) was prepared. Then, the master mix reaction tube was vortex and spun. After this, the master mix was aliquoted into individual tube. During the preparation step before incubation, the enzymes were kept on ice.

After the incubation, the result was checked via gel electrophoresis using 3% agarose gel. The 3µl PCR- RFLP products were each mixed with 1 µL loading dye and loaded into each well. Electrophoresis was at 65 Volt for 95 minutes. After gel electrophoresis, the gel was immersed in the Ethidium Bromide for 30 minutes to enable visualization under UV light.

CHAPTER 3

RESULTS and DISCUSSION:

Figure 2 was the PCR analysis of samples analyzed. Fragments which were about 600bps were obtained by using the 16S rRNA primers. All the eight fragments were observed at the expected site.

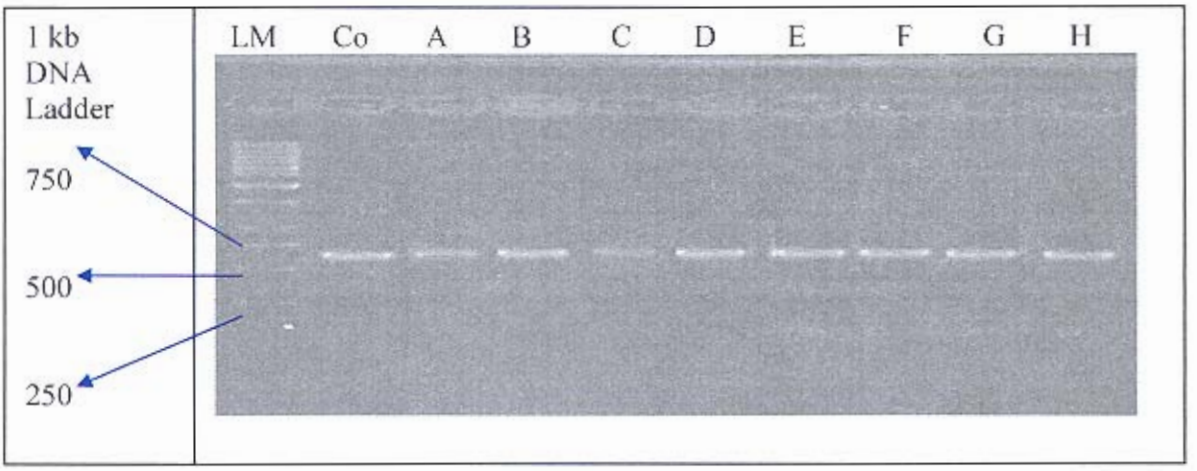

FIGURE 3: Agarose gel electrophoretic analysis of purified PCR product.
LM: Ladder Marker, Co: Control, A: TK001, B: MTA96328, C: PR055, D: PR056, E: PR057, F: PR058, G: PR059, H: F#21.

The partial 16S rRNA gene of the PCR- amplified products, which is about 600 bps, from the eight individual *Myotis ridleyi* was shown in Figure 3. The intensity of each band was different. The differences arising could be due to the quantity of the DNA. This factor would affect the concentration of the PCR products obtained. Among the eight individuals of *Myotis ridleyi*, seven from Sabah (five from Poring, one from Taman Kinabalu, one from Danum Valley) and one from Gunung Serapi, Sarawak. Only one individual from Sarawak was available in this study because there was only one sample preserved in the FSTS museum. According to previous record on the distribution of *Myotis ridleyi* in IndoMalayan region and Borneo (Corbet and Hill,